Increase in ubiquitin–protein conjugates concomitant with the increase in proteolysis in rat skeletal muscle during starvation and atrophy denervation

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The rapid loss of skeletal-muscle protein during starvation and after denervation occurs primarily through increased rates of protein breakdown and activation of a non-lysosomal ATP-dependent proteolytic process. To investigate whether protein flux through the ubiquitin (Ub)–proteasome pathway is enhanced, as was suggested by related studies, we measured, using specific polyclonal antibodies, the levels of Ub-conjugated proteins in normal and atrophying muscles. The content of these critical intermediates had increased 50–250% after food deprivation in the extensor digitorum longus and soleus muscles 2 days after denervation. Like rates of proteolysis, the amount of Ub–protein conjugates and the fraction of Ub conjugated to proteins increased progressively during food deprivation and returned to normal within 1 day of refeeding. During starvation, muscles of adrenalectomized rats failed to increase protein breakdown, and they showed 50% lower levels of Ub–protein conjugates than those of starved control animals. The changes in the pools of Ub-conjugated proteins (the substrates for the 26S proteasome) thus coincided with and can account for the alterations in overall proteolysis. In this pathway, large multi-ubiquitinated proteins are preferentially degraded, and the Ub–protein conjugates that accumulated in atrophying muscles were of high molecular mass (> 100 kDa). When innervated and denervated gastrocnemius muscles were fractionated, a significant increase in ubiquitinated proteins was found in the myofibrillar fraction, the proteins of which are preferentially degraded on denervation, but not in the soluble fraction. Thus activation of this proteolytic pathway in atrophying muscles probably occurs initially by increasing Ub conjugation to cell proteins. The resulting accumulation of Ub–protein conjugates suggests that their degradation by the 26S proteasome complex subsequently becomes rate-limiting in these catabolic states.

INTRODUCTION

Muscle size is determined by the balance between the rates of protein synthesis and degradation. Accelerated protein degradation is an important factor contributing to the loss of muscle weight in various physiological and pathological conditions, including starvation [1–3] and denervation atrophy [4]. Like other cells, skeletal muscle contains multiple intracellular proteolytic systems [5–7], and it remains unclear which degradative systems are responsible for the accelerated proteolysis in these and other catabolic states. Although intralysosomal proteolysis rises in muscle and other cells when the supply of amino acids and insulin falls [5], this response cannot account for most of the increased proteolysis or the breakdown of myofibrillar components in food deprivation [8] or denervation atrophy [4]. Our previous studies using isolated rat muscles instead indicate that a non-lysosomal ATP-dependent process is activated in response to food deprivation and denervation. Moreover, the rise in the ATP-dependent process correlates precisely with the overall enhancement of protein degradation and seems to account for most of the accelerated proteolysis in these atrophying muscles [9].

The major non-lysosomal ATP-dependent proteolytic system in eukaryotic cells involves ubiquitin (Ub) and the proteasome particle (for reviews, see refs. [10,11]). In this pathway, multiple molecules of the polypeptide Ub are covalently linked to lysine residues on the proteins to be degraded. This conjugation process requires multiple enzymes and ATP [12–14], and marks the proteins for rapid degradation by the 26S (1500 kDa) proteasome complex [15–18], the functioning of which also requires ATP. However, some of these Ub-conjugated proteins can also be deubiquitinated by isopeptidases, which release free Ub moieties from the protein [19,20]. Recent findings suggest that in skeletal muscle that is atrophying as the result of disuse [21], denervation or food deprivation [22], this Ub–proteasome-dependent proteolytic pathway is activated. For example, the levels of RNA for Ub [12] and proteasome subunits and the total Ub content of the muscles increase [22] in parallel with increases in overall proteolysis and in the ATP-dependent process [9]. Thus denervation and starvation lead to adaptations in the muscle that should enhance or maintain the capacity of the Ub–proteasome-dependent pathway.

Such observations, however, do not prove increased flux of proteins through this pathway, nor do they indicate which steps in this degradative pathway may be altered or rate-limiting in atrophying muscles. Increased expression of Ub in these atrophying muscles *per se* is unlikely to accelerate the degradation of proteins by the pathway, because the levels of free Ub in normal muscles should be saturating the Ub-activating enzyme (E1) [23,24]. Therefore it seems more likely that the increase in Ub in atrophying muscles occurs largely in the form of Ub–protein conjugates. Increasing the levels of ubiquitinated proteins under these conditions should accelerate proteolysis by increasing the availability of substrates for the 26S proteasome complex.

Abbreviations used: Ub, ubiquitin; EDL, extensor digitorum longus.

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Accordingly, a rise in the levels of Ub–protein conjugates has been observed in inactive leg muscles atrophying in response to hind-limb suspension [24]. To clarify the mechanisms leading to enhanced rates of proteolysis during starvation or denervation, we have examined carefully the levels of Ub-conjugated proteins and free Ub in rat skeletal muscles under a variety of conditions in which overall rates of proteolysis and the ATP-dependent degradative process had been found to rise. As the increase in proteolysis observed on starvation can be prevented by adrenalectomy or can be reversed by refeeding, we have investigated whether these treatments reduce the accumulation of Ub–protein conjugates. In addition, we have characterized the nature and size distribution of the conjugates that accumulate in muscle after denervation, since multiple ubiquitinated proteins are degraded preferentially by the 26S proteasome [25]. Finally, because myofibrillar components, which comprise most of the proteins in muscle, appear to be degraded selectively during food deprivation [26,27] and in denervation atrophy [4], we have tested whether the increases in ubiquitinated proteins occur primarily in this fraction of the muscle.

EXPERIMENTAL

Animals

Male CD rats, 70–90 g when killed (Charles River Laboratories, Wilmington, MA, U.S.A.), were fed *ad libitum* with Purina Lab Chow for at least 3 days before any experimental procedures. Because the absolute levels of Ub–protein conjugates in cells are very sensitive to environmental influences [23], control and experimental animals were always chosen from a common pool and studied in parallel. Denervation of the hind limb of one leg was performed by removing a 1 mm segment of the sciatic nerve 1 cm above the popliteal fossa [4]. The muscle in the contralateral limb was used as a control. Preliminary studies have shown that similar results are obtained whether or not a sham-operation is carried out on the opposite leg (results not shown). Adrenalectomy was performed as previously described [28]. After 3 days (to allow clearance of adrenal hormones and proteins induced by these hormones), the rats were used in experiments. Control rats received a sham operation. Animals were killed by cervical dislocation at various times after food deprivation, refeeding or denervation, and tissues rapidly removed and frozen in liquid nitrogen or, in some experiments, fractionated as described below.

Measurement of free and conjugated Ub

Detailed methods for measuring free and conjugated Ub have been described previously [23,29]. Antibodies specific for free and conjugated species were produced by immunizing rabbits with native [30] or denatured [29] Ub respectively fused to bovine γ-globulin. Muscles were solubilized in the presence of SDS and protease inhibitors as previously described [23]. After centrifugation to remove insoluble material, protein was quantified by the Lowry method with BSA as standard [31]. Free Ub was measured by solution radioimmunoassay using anti-(free Ub) antibody. Ubiquitinated histone H2A was used as a control to confirm the specificity of the assay for free Ub [30]. Quantification of conjugated Ub was by a solid-phase immunochemical method. Samples were dot-blotted on to nitrocellulose membranes. The membranes were probed sequentially with anti-(Ub–protein conjugate) antibody and ^{125}I-Protein A [23,29]. After autoradiography, the films were quantified by densitometry. Specificity for conjugated Ub was confirmed by the absence of signal for free Ub dot-blotted on to the same membrane. Ub purified from bovine erythrocytes and conjugates produced in rabbit reticulocyte extracts were used as standards [29].

Separation of soluble and myofibrillar fractions

To determine whether ubiquitination of soluble or myofibrillar components increases following denervation, gastrocnemius muscles were subjected to subcellular fractionation. The gastrocnemius was studied because the much larger muscle size permits reproducible fractionation and the muscle also undergoes atrophy following denervation [32]. Procedures were carried out on ice or at 4 °C. Muscles were minced with a razor blade and then homogenized with a motor-driven Dounce homogenizer in 10 mM Tris/maleate, pH 7.0, containing 10 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM L-tosylamido-2-phenylethylchloromethyl ketone, 1 mM tosyl-L-lysine chloromethyl ketone and 5 mM *N*-ethylmaleimide. The homogenate was centrifuged at 1500 *g* for 10 min. To prepare the myofibrillar fraction [33], the resulting pellet was resuspended in buffer containing 1 % Triton and then twice more with buffer without Triton before being solubilized in 10 mM Tris/maleate, pH 7.0, containing 1 mM L-tosylamido-2-phenylethylchloromethyl ketone, 1 mM tosyl-L-lysine chloromethyl ketone, 5 mM *N*-ethylmaleimide and 2 % SDS for quantification. The soluble fraction was prepared by centrifugation at 10000 *g* for 10 min and then at 100000 *g* for 1 h. Ub–protein conjugates were then measured in the final supernatant.

To determine whether there were increases in specific ubiquitinated proteins, the myofibrillar fractions were analysed by immunoblotting with anti-Ub antibodies. Pooled aliquots of the samples derived from individual muscles were electrophoresed on SDS/10 % polyacrylamide gels and electroblotted on to nitrocellulose in solution comprised of 20 mM Tris base, 150 mM glycine, 20 % methanol and 0.02 % SDS (150 mA, 16 h). The membrane was probed sequentially with anti-Ub antibody (0.5 μg/ml) and goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad) (diluted 1:250000) and developed using a chemiluminescent assay (ECL, Amersham) and exposed to film. The autoradiographs were quantified using an LKB Ultroscan XL densitometer and the associated Gelscan software. To confirm that similar amounts of protein from innervated and denervated samples were loaded on the gel, duplicate gels were stained with Amido Black and quantified with the densitometer.

Statistical analysis

Means of muscle samples from different groups of animals were analysed using Student's unpaired *t* test. Means of samples from contralateral legs were compared using Student's paired *t* test. Similar effects of starvation, denervation and adrenalectomy on Ub–protein conjugates were seen in at least two independent experiments each using at least six animals. Control and starved animals used in this study were siblings and studied in parallel in each experiment. As previously noted [23], the absolute levels of Ub–protein conjugates (and proteolysis) vary significantly from day to day. Therefore comparisons of data between different groups of rats or of measurements from different experiments are not valid and were avoided.

RESULTS

Effects of fasting and denervation on different Ub pools

Using antibodies that specifically recognize Ub-conjugated proteins (to the exclusion of free Ub) [29], we observed an

Table 1 Changes in free, protein-bound and total Ub in muscles atrophying due to fasting or denervation

EDL muscles from fed rats and those deprived of food for 2 days (A) and soleus muscles from innervated and contralateral denervated rats (2 days after section of the sciatic nerve) (B) were studied and levels of Ub and Ub–protein conjugates measured as described in the text. Values are means ± S.E.M. for three to seven muscles. Differences that are significant are indicated: * $P < 0.005$; † $P < 0.001$; ‡ $P < 0.02$; § $P < 0.01$.

Condition	Muscle weight (mg)	Ub (pmol/mg of protein)		Ub–protein conjugates		
		Total	Free	pmol of Ub/mg of protein	pmol of Ub/muscle	% of total Ub
(A)						
Fed	39 ± 2	15.9 ± 1.6	12.4 ± 1.5	3.5 ± 0.5	33.6 ± 5.7	22
Starved	26 ± 2	25.8 ± 1.8	15.9 ± 1.4	9.9 ± 0.8	63.1 ± 2.7	38
Difference	13 ± 2*	9.9 ± 2.5‡	3.5 ± 2.1	6.4 ± 1.0*	29.5 ± 6.9§	16
(B)						
Innervated	33 ± 2	26.8 ± 1.9	16.8 ± 1.3	10.0 ± 0.7	35.3 ± 1.5	37
Denervated	27 ± 2	51.2 ± 2.3	25.3 ± 1.2	25.8 ± 1.3	72.9 ± 6.8	50
Difference	6 ± 1†	24.4 ± 3†	8.5 ± 1.9*	15.8 ± 1.3†	37.6 ± 6.0†	13

increase in the levels of Ub–protein conjugates in the pale extensor digitorum longus (EDL) muscle from animals 2 days after food deprivation (Table 1). This muscle was studied because pale muscles show greater atrophy after food deprivation [1] or treatment with adrenal steroids [34], and we have measured the ATP-dependent proteolytic process in incubated EDL muscles at different times after food deprivation [9]. Using antibodies that specifically recognize free Ub [29], no significant increase was detected in response to food deprivation. On denervation, the soleus undergoes marked atrophy, losing 20% of its wet or dry weight in 2 days [4]. In the denervated soleus, we also detected significant increases in Ub–protein conjugates as well as an increase in free Ub (Table 1).

During starvation and following denervation, atrophying skeletal muscles showed increases in their total content of Ub (Table 1) [22]. The percentage of total cell Ub that is conjugated to cell proteins also increased during both starvation and denervation atrophy. In both catabolic states, the increases in Ub–protein conjugates accounted for approx. 65% of the increase in total Ub. These findings indicate a net increase in Ub conjugation to cell protein in atrophying muscles. As was previously noted in the fed state [23], levels of both free and conjugated Ub are higher in the red soleus muscle than in the pale EDL muscle. This difference correlates with a greater rate of ATP-dependent proteolysis in the red soleus (H. Q. Han, R. Medina, I. C. Kettelhut, K. Furuno and A. L. Goldberg, unpublished work).

To examine more carefully the relationship between the increases in degradative rates and Ub–protein conjugate levels, we studied the time course of the changes in Ub–protein conjugates in the muscle during food deprivation, refeeding and denervation atrophy. After food deprivation, Ub–protein conjugates accumulated progressively with the duration of the food-deprivation period, and, after refeeding, their levels returned to control values within 1 day (Figure 1). In this experiment, the increase in Ub–protein conjugates was not as great as was observed in the experiments described in Table 1. In fact, as noted previously [23], the basal levels of Ub–protein conjugates in muscle vary widely between experiments, which is not surprising as many physiological factors [e.g. food intake, contractile activity, glucocorticoids (see below)] can affect Ub conjugation. Despite this day-to-day variability, the finding of an accumulation of Ub–protein conjugates was highly reproducible and statistically significant ($P < 0.001$ to $P < 0.01$) when compared with the levels in control tissues of sibling animals, measured simultaneously. Moreover, the time course of changes in Ub–protein conjugates resembled closely the changes reported previously in rates of proteolysis and in levels of polyUb and proteasome mRNA in response to food deprivation and refeeding [22] (Figure 1). In response to denervation, the levels of Ub–protein conjugates in the soleus also increased. This effect was statistically significant only at 2 days of denervation, just after the rise in proteolysis was demonstrable (Figure 2).

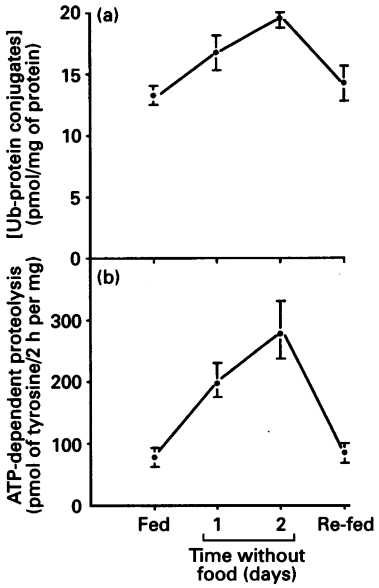

Figure 1 Effects of food deprivation and refeeding on levels of Ub–protein conjugates in EDL muscles

Muscles were removed from fed controls, animals deprived of food for 1 or 2 days, and those re-fed for 1 day (after 2 days without food), and (**a**) conjugates were quantified. The levels of Ub-conjugates at 2 days of fasting were significantly different from those in the fed and re-fed animals ($P < 0.001$). Each value is the mean ± S.E.M. from muscles of five to six animals. (**b**) Rates of ATP-dependent proteolysis in muscles from rats treated similarly are shown for comparison [12].

The capacity to increase muscle proteolysis and the ATP-

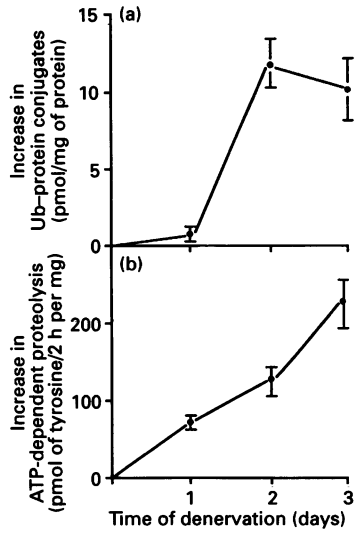

Figure 2 Effects of denervation on levels of Ub–protein conjugates in soleus muscles at different times after unilateral section of the sciatic nerve

Soleus muscles were removed from the innervated and denervated legs and Ub–protein conjugates quantified. The difference in levels of Ub–protein conjugates between the innervated and denervated legs is shown (**a**). Each value is the mean ± S.E.M. obtained from muscles of five to six animals. Differences between means of innervated and denervated muscles were significant ($P < 0.005$) at days 2 and 3 after section of the sciatic nerve. Rates of ATP-dependent proteolysis from similarly treated muscles are shown for comparison (**b**) (H. Q. Han, R. Medina, I. C. Kettelhut, K. Furuno, and A. L. Goldberg, unpublished work).

Table 2 Effect of adrenalectomy on the levels of Ub–protein conjugates and the rates of ATP-dependent proteolysis in muscle during starvation

Sham-operated normal rats and adrenalectomized animals were starved for 1 day, and the levels of Ub–protein conjugates in their EDL muscles were measured as described in the Experimental section. Rates of ATP-dependent proteolysis were measured in similar muscles during incubation *in vitro* [28]. The ATP-dependent proteolytic process was measured in muscles as described [9]. The muscle from one limb was incubated in Ca^{2+}-free Krebs–Ringer bicarbonate buffer containing 5 mM glucose, 0.5 mM cycloheximide, 0.1 unit/ml insulin, 0.17 mM leucine, 0.1 mM isoleucine, 0.2 mM valine, 10 mM methylamine and 50 μM E64 to suppress lysosomal and Ca^{2+}-dependent proteolysis. The paired contralateral muscle was depleted of ATP by incubation in the same medium except that it also contained 0.5 mM dinitrophenol and 5 mM 2-deoxyglucose and no glucose. The non-lysosomal ATP-dependent component of proteolysis was calculated as the difference between the rates of protein breakdown of the paired muscles. Values are means ± S.E.M. of six animals. The differences are all significant: * $P < 0.05$; † $P < 0.001$; ‡ $P < 0.005$.

	Muscle weight (mg)	Ub–protein conjugates		ATP-dependent proteolysis (pmol of tyrosine/2 h per mg)
		pmol/mg of protein	pmol/ muscle	
Starved normal	28 ± 1	27.9 ± 1.7	150 ± 20	433 ± 18
Starved adrenalectomized	32 ± 1	15.6 ± 1.2	81 ± 6.5	316 ± 24
Difference	4 ± 1*	12.3 ± 2.0†	69 ± 18‡	117 ± 10†

dependent proteolytic process during starvation are impaired in adrenalectomized animals [28,35]. As a consequence, starved adrenalectomized rats lose less muscle mass than normal animals during food deprivation (Table 2). To test whether the rise in Ub–protein conjugates is linked to the rise in ATP-dependent proteolysis, we compared the Ub–protein conjugate pools in muscles of adrenalectomized and control rats during starvation. One day after food deprivation, the muscles of adrenalectomized animals demonstrated a 44% lower content of Ub–protein conjugates than tissues of starved normals (Table 2) in accord with their reduced proteolytic capacity. These findings further suggest that the build-up of Ub–protein conjugates during starvation leads to the rise in proteolysis.

Table 3 Accumulation of Ub–protein conjugates in the myofibrillar fraction, but not the soluble fraction, after denervation

Previous results showed that the gastrocnemius, like the soleus, undergoes atrophy in response to denervation, but the much larger size of the gastrocnemius allows fractionation and further analysis. Gastrocnemius muscles from innervated and denervated legs were removed 2 days after section of the sciatic nerve and fractionated by differential centrifugation. The contents of Ub–protein conjugates in the soluble and myofibrillar fractions were then assayed in the usual manner. Values are means ± S.E.M. from six animals. Differences that were significant are indicated: * $P < 0.001$.

	Content of Ub–protein conjugates			
	Soluble fraction		Myofibrillar fraction	
	pmol/mg of protein	pmol/ muscle	pmol/mg of protein	pmol/ muscle
Innervated	11.8 ± 0.9	67 ± 7	3.1 ± 0.3	68 ± 7
Denervated	14.6 ± 2.5	86 ± 9	7.0 ± 0.4	143 ± 13
Difference	2.8 ± 1.0	19 ± 10	3.9 ± 0.4*	75 ± 12*

Ub–protein conjugates in soluble and myofibrillar fractions after denervation

As most of the muscle protein loss during starvation [26,27] and after denervation [4] occurs from the myofibrillar fraction, and as breakdown of these components increases disproportionately, we examined whether the increase in Ub–protein conjugates occurred preferentially in the myofibrillar or soluble fractions of the muscle. Gastrocnemius muscle was used for these studies because its greater mass allowed more reproducible fractionation than did the soleus. Also, the gastrocnemius undergoes atrophy following section of the sciatic nerve in a manner similar to the soleus [32]. Denervation was studied because the absolute increases in Ub–protein conjugates following denervation were larger than during starvation (Figures 1 and 2), and also because denervation allowed a more sensitive detection of any changes than did food deprivation (i.e. comparison of the contralateral atrophying and normal muscles eliminated animal to animal variations). In the innervated muscle, the level of ubiquitination per mg of protein was higher in the soluble than in the myofibrillar fraction, which is consistent with the higher turnover rate of soluble protein [36]. After denervation, the soluble fraction (i.e. the supernatant produced by centrifugation at 100000 *g*) did not show a significant increase in Ub–protein conjugates. However, the myofibrillar fraction showed a clear (126%) increase in these conjugates (Table 3), which can account for the overall increase in Ub–protein conjugates in the muscle.

The number of Ub moieties conjugated to a protein vary widely, and larger Ub–protein conjugates tend to be digested more rapidly by the proteasome [25]. To determine whether the increase in ubiquitinated proteins was general or restricted to conjugates of a specific size (Figure 3), these myofibrillar fractions were analysed further by immunoblotting. In the innervated muscle, diffuse staining by the antibody was detected in the high-molecular-mass region (> 100 kDa) of the gel, which accounted for approx. 60% of the total staining. As electroblotting of high-molecular-mass proteins to nitrocellulose is quite inefficient, the

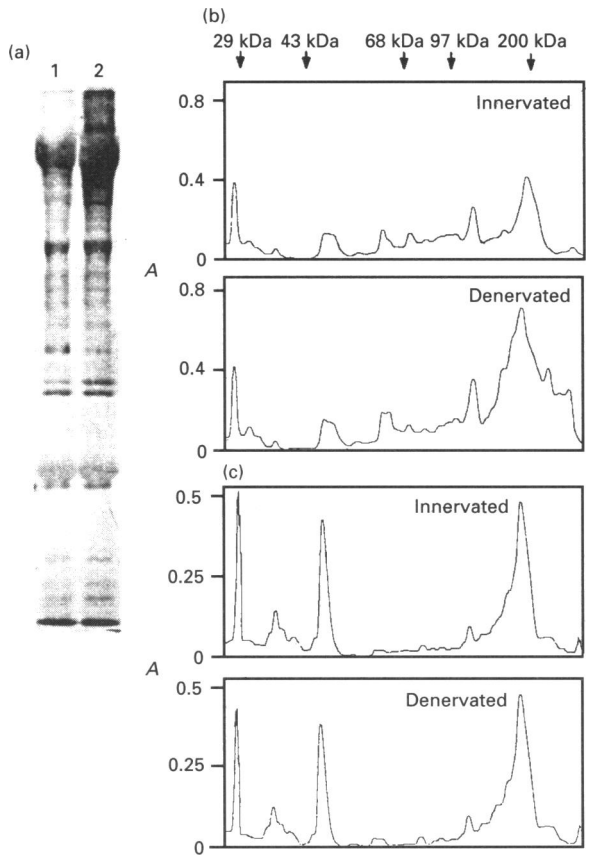

Figure 3 Composition of ubiquitinated proteins in the myofibrillar fraction following denervation

(**a**) Autoradiograph of anti-Ub immunoblot of 100 μg of myofibrillar proteins from innervated (lane 1) and denervated (lane 2) gastrocnemius muscle. (**b**) Scanning densitometry of the blot in (**a**). (**c**) Scanning densitometry of a duplicate gel stained with Amido Black.

degree of immunoreactivity in this region must be markedly underestimated. Therefore the high-molecular-mass conjugates must represent most of the ubiquitinated proteins in the myofibrillar fraction.

The proteins from the denervated muscle also showed diffuse staining in this high-molecular-mass region. However, the amount of high-molecular-mass conjugates in the sample from the denervated muscle was 2.4-fold greater than in the control muscle (Figure 3b). Samples from both the innervated and denervated muscle also showed minor staining of protein bands between approx. 20 and 66 kDa (Figure 3a). However, staining in this area did not increase upon denervation, and some of the bands may represent non-specific binding, because prominent protein bands are detectable at similar positions of a duplicate gel stained with Amido Black (Figures 3b and 3c). Control studies of this duplicate gel (Figure 3c) confirmed that similar amounts of protein were loaded on the gel, and that the overall pattern of proteins in the myofibrillar fraction did not change on denervation. The Amido Black-stained gel revealed major protein bands of approx. 200, 46 and 20 kDa consistent with sizes of myosin heavy chain, actin and myosin light chains, indicating that the myofibrillar fractions were relatively pure. Soluble proteins were also analysed by immunoblotting with the anti-Ub antibody. As expected, the total staining of the soluble proteins was similar in the innervated and denervated samples, as was the distribution of the stain (results not shown).

DISCUSSION

The demonstration that levels of ubiquitinated proteins increase in muscle during starvation and after denervation provides strong evidence that the Ub-dependent pathway plays an important role in the protein loss that occurs in these atrophying muscles. These increases in the content of Ub–protein conjugates coincided with the overall acceleration of the ATP-dependent proteolytic process after food deprivation and denervation, and both parameters fell rapidly on refeeding. Moreover, adrenalectomy, which blocks the rise in proteolysis that occurs during starvation, reduced the increase in Ub–protein conjugate levels. We have demonstrated increases in the levels of mRNA encoding Ub and in the total Ub content of the muscles [22] on starvation and denervation. As shown here, most of this increase in Ub is found covalently linked to proteins. Thus Ub production appears to increase in conjunction with a stimulation of Ub conjugation to cell proteins, perhaps as a compensation for an increased utilization of Ub. On heat shock, Ub production also rises as a consequence of increased Ub conjugation to proteins [37].

Myofibrillar proteins, which comprise the bulk of muscle mass, are normally long-lived components, but they are the source of most of the proteins degraded on denervation [4] and during starvation [26,27]. It is noteworthy that the increase in Ub–protein conjugates during food deprivation occurs in the myofibrillar fraction, but not in the soluble fraction of the muscle. Ub–protein conjugates are generally soluble [29]; therefore it is unlikely that their appearance in the myofibrillar fraction is an artifact caused by precipitation. Immunoelectron microscopic studies have also suggested staining of myofibrils by antibodies to Ub–protein conjugates [23]. These results offer further support to previous conclusions that myofibrillar proteins are degraded by a non-lysosomal [4,8] Ca^{2+}-independent mechanism, probably the ATP–Ub-dependent system [9].

Our results also indicate that most of the ubiquitinated proteins in the myofibrillar fraction are of high molecular mass (> 100 kDa). Such large conjugates have been found *in vitro* to be preferentially degraded by the 26S (1500 kDa) proteasome complex [25]. Furthermore, the increase in the ubiquitinated proteins in the myofibrillar fraction following denervation occurred predominantly in these large conjugates, whereas the amounts of the smaller ubiquitinated proteins did not change. Such high-molecular-mass Ub–protein conjugates also accumulate rapidly (within an hour) following heat shock of chick embryo fibroblast [37] and in yeast (D. H. Lee, M. Sherman and A. L. Goldberg, unpublished work), conditions under which Ub-dependent proteolysis also rises. However, the response in atrophying muscles is not simply a manifestation of this stress response, as there is no increase in expression of heat-shock genes in the denervated muscle [22]. As the changes in Ub–protein conjugate levels coincided with altered rates of ATP-dependent proteolysis in the muscle, it seems very likely that these high-molecular-mass conjugates represent the multiubiquitinated proteins destined for degradation.

These data together indicate that denervation and food deprivation lead to a net increase in the conjugation of Ub to muscle proteins. (As the flux of proteins through the pathway does not fall, the accumulation of intermediates must reflect a stimulation of Ub conjugation to cell proteins.) This process involves multiple steps catalysed by the Ub-activating enzyme (E1), Ub-carrier proteins (E2s) and, with most substrates, Ub-protein ligases [11,38]. The enzymes involved in Ub conjugation are highly

specific for the substrate, and this process is highly regulated. It is unlikely that the increased expression of Ub observed during starvation and after denervation [22] would by itself cause the increased Ub conjugation, as the levels of free Ub and ATP are saturating for the Ub-activating enzyme under normal conditions (e.g. muscles of fed animals) [23,24]. Increased activity of Ub-activating enzyme is also unlikely by itself to be responsible for the increased Ub conjugation, because this enzyme is not rate-limiting and normally can transfer Ub to a large excess of Ub-carrier proteins [14]. Thus the rise in Ub conjugation probably takes place through enhanced activity of the Ub-carrier proteins and/or the Ub–protein ligase, or by a modification of cell proteins, which increase their susceptibility to these enzymes. Both types of adaptation are well documented in yeast, for example, during heat shock, when there is rapid Ub-dependent degradation of damaged proteins [11,38]. In fact, recent observations indicate that mRNA levels of a Ub-conjugating enzyme (E2) are also increased in skeletal muscle during fasting [39], which suggests that conjugation of Ub to proteins is an initial rate-limiting step in the degradative pathway. It is also theoretically possible that the activity of a Ub-conjugate isopeptidase, the enzyme that can dissociate the polyUb chains, could decrease during atrophy and lead to the increased levels of conjugates. Little is known about this step, and no such regulation has yet been demonstrated in other cells. It seems highly unlikely that Ub–protein conjugates accumulate as the result of a decrease in their degradation by the 26S complex, as, in these same muscles, ATP-dependent breakdown of cell proteins [9] increases, as do mRNA levels for several subunits (C-2, C-5, C-7 and C-8) of the 20S proteasome [22], which comprise the core of the 26S proteolytic complex [40].

The simplest and most likely model to explain the increased rate of protein degradation is that denervation and food deprivation lead to enhanced Ub conjugation to proteins, and the resulting enlarged pool of conjugates provides more substrates for the 26S proteasome complex [10,11,40]. The present findings all strongly support such a model. The rates of degradation in the muscle were not directly proportional to the absolute levels of ubiquitinated proteins; a significant increase in proteolysis was demonstrable 1 day after nerve section, whereas the increase in Ub–protein conjugates was only demonstrated a day later. Such minor discrepancies may be due to the degree of variation inherent in such measurements. However, the flux of substrates through a pathway is not necessarily proportional to the levels of an intermediate, and a simple proportionality between the overall rates of proteolysis and the Ub–protein conjugate levels would not be expected for several other reasons. First, the ubiquitinated protein fraction is very heterogeneous, and includes different cell proteins that vary widely in their degree of ubiquitination and rates of proteolysis. For example, monoubiquitinated proteins are probably not substrates for degradation, whereas the large multiubiquitinated proteins are digested very rapidly. Also, the rates of degradation of different proteins with the same degree of ubiquitination vary. Secondly, not all of the ATP-dependent proteolysis observed may be Ub-dependent; such a proteolytic activity has been observed in skeletal muscle [41].

It also seems likely, on the basis of these findings, that regulation of this pathway also occurs at a step after formation of Ub–protein conjugates. In fact, the accumulation of conjugates 2 days after denervation and food deprivation strongly suggests that the hydrolysis of Ub-conjugated proteins has become the rate-limiting step in the degradative pathway at these times. Moreover, since the levels of mRNAs encoding proteasome subunits increase, the cell's capacity to digest Ub-conjugated proteins appears also to be regulated by nutritional factors and innervation. Since multiple steps in the ATP–ubiquitin pathway in muscle appear to be regulated, the close association found between levels of Ub–protein conjugates and degradative rates appears quite remarkable. Increased Ub–protein conjugates have also been observed within the intersegmental muscles of insects undergoing programmed cell degeneration (A. L. Haas and L. Schwartz, unpublished work) and, recently, in rat skeletal muscle after administration of tumour necrosis factor [42] or tumour transplantation [43], conditions under which proteolysis rises. Thus a common programme for muscle atrophy appears to exist in many physiological and pathological conditions, and the increases in Ub–protein conjugates may be a useful marker of accelerated muscle proteolysis in various catabolic states.

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